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13. ABSTRACT *(Maximum 200*

It is proposed to study the metabolism of intact breast cancer cells using non-invasive magnetic resonance spectroscopy (MRS) methods. Magnetic resonance imaging (MRI) has been shown to have some value in assessing breast lumps, but lacks specificity. MRS can in principle provide this specificity. In order to evaluate the potential of MRS, systematic studies will be carried out on cell lines selected for the progressive estrogen independent phenotype and the effects of hormones and tamoxifen.

The cell studies will provide basic information on the metabolic profile of different BC cell lines in the progression from estrogen and drug sensitive to estrogen independent and drug resistant phenotypes. Cells are embedded in gels and are examined in the MR spectrometer while undergoing perfusion. Previous studies have mainly used carbohydrate (agarose) gels. But, based on preliminary results, we will use a protein gel (Matrigel) for which the cells have surface receptors, so that their metabolism can be monitored while they are proliferating. This allows the effects not only of different phenotypes, but also of hormones, and anti-estrogens such as tamoxifen, as well as certain drugs and drug combinations to be evaluated. Both ^{31}P and proton MR methods will be applied. The results will be of significance not only to understanding the fundamental biological processes involved, but also to the observation of *in vivo* MRS of breast lumps.

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FOREWORD

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Joel Sloan 7/24/96

PI Signature Date

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1. INTRODUCTION

A. Background

We are investigating the metabolism of intact breast cancer cells using MRS methods in order to shed light on the processes involved in the development and treatment of breast cancer.

Magnetic resonance techniques have become important in clinical imaging, but perhaps less well known is the fact that MRS spectroscopy can be used as a research tool to study the metabolism of isolated intact cells (Daly and Cohen, 1989). Such studies can provide important information on biochemical processes, and can be used for the identification of signals and the understanding of metabolic processes in vivo.

Perfused intact cells represent possibly the best approach to the non-invasive study of metabolism. In contrast to the in vivo situation, the cells are homogeneous, particularly when grown in culture conditions. Such ex vivo 31P MRS studies have provided information on normal cellular energetic status, substrate utilization and metabolic pathways, phospholipid pathways, intracellular pH changes and membrane permeability; using these methods, significant metabolic differences between cell lines have been delineated and the effects on metabolism following manipulation with nutrients, hormones, drugs, growth factors, and hyperthermia have been monitored (for a recent review see Cohen et al, 1995).

A variety of methods for restraining cells for MRS studies of metabolism are currently available. An appropriate technique uses agarose threads; the advantages of this method are: (a) it is a simple, inexpensive and quick technique; (b) a large number of cells can be maintained in good metabolic status for prolonged experiments (24 to 36 h); (c) the matrix occupies a relatively small volume; (d) effects of metabolite precursors and derivatives, drugs, and physical insults on metabolism can be detected; (e) both anchorage-dependent and -independent cells can be studied. Detailed studies of cell growth and viability (using trypan blue exclusion and cell counting), microscopy, and the measurement of diffusion constants of metabolites (ATP, glucose) and protein content, were carried out using cells in agarose threads (Foxall and Cohen, 1983; Foxall et al, 1984; Knop et al, 1984; Lyon et al, 1986; Kaplan et al, 1990).

Agarose is a carbohydrate gel and in the agarose method cells are "trapped" in the threads and are simply removed by gentle pipetting. Matrigel is a natural basement membrane and is a protein gel for which breast cancer cells have receptors. It is an extremely open gel that is used extensively for studies of cancer cells (Kleinman et al, 1986, 1993), Dispase is used to dissolve matrigel in order to measure cell densities (Daly et al, 1988). It should also be emphasized that a comparison of the same cell line in both the matrigel cell threads and in tumor xenografts in nude mice gave almost identical spectra (Daly et al, 1988). We have chosen the matrigel thread method as the most effective one for this work. Therefore, the bulk of this proposal is devoted to 31P MRS studies of perfused intact breast cancer cells embedded in matrigel threads.

In order to understand the role of ER status in breast cancer cells Clarke et al. (1989, 1990), have developed special cell lines (Table 1). The cell lines to be studied in this work are all variants that have been isolated and extensively characterized, and exhibit specific phenotypic changes that reflect critical characteristics of the progressed phenotype. The ability to detect specific metabolic changes associated with phenotypic changes is substantially increased, since these cells were all derived from the same parental cell line (MCF7).

In breast cancer cells and other malignant cell lines, some of the cellular effects of "anti microtubule" drugs are modulated by the action of steroids, estrogens and antiestrogens, and vice versa. For example, colchicine was reported to inhibit translocation of estradiol receptors and the synthesis of progesterone receptors in MCF-7 cells (Parikh et al, 1987); and to sensitize the activation of large conductance chloride channels in fibroblasts upon exposure to extracellular antiestrogens (Hardy and Valverde, 1994). Tamoxifen increases the cytotoxicity of vinblastine in MCF-7 variants which express gp170 (Leonessa et al, 1994) and reversed vinblastine resistance in the mdr1 transfected lung cancer cell line, S1/1.1 (Kirk et al, 1993). The antiestrogen toremifene, 'resensitize' MDR MDA-MB-231-A1, an estrogen receptor negative breast cancer cells, to vinblastine (Koester et al, 1994). The estrogen metabolite, 17 beta-estradiol glucuronide modulate resistance to taxol and vinblastine in the Dx5 MDR sarcoma cell line (Gosland et al, 1993). It was recently reported that a metabolite of estradiol, 2-methoxyestradiol, inhibits tubulin polymerization by binding to the colchicine binding site on tubulin dimer (Cushman et al, 1995).

Among the anti-cancer drugs that will be examined are taxol, vincristine and colchicine. Taxol (Paclitaxel), a new promising anticancer drug, has recently been approved for the treatment of refractory ovarian cancer, and is showing promising activity in malignant melanoma, breast cancer, and lung cancer (see: Paclitaxel in Cancer Treatment; edited by Mcguire and Rowinsky, 1995). Taxol has demonstrated substantial single agent activity against both minimally pretreated and resistant metastatic breast cancer (Wong and Henderson, 1994). Taxol, vincristine and colchicine are anti mitotic drugs that interfere with the assembly process of microtubules. Microtubule functions in cells are highly complex. Aside from serving as passive skeletal supports for the organization of the cytoplasm, their remarkable polymerization dynamics are critical to many of their functions. Drugs like taxol, vincristine and colchicine interacts with microtubule ends and surfaces and modulate the polymerization dynamics. Although all of these drugs bind to tubulin, they exert distinct effects on the protein organization in the cell. In the presence of taxol highly organized bundles of microtubules are formed, while vinblastine induces tubulin self-association to paracrystal formations. Colchicine interacts with tubulin subunits and inhibits microtubule assembly and mitosis (for review see: Wilson and Jordan, 1994). These drugs inhibit cell proliferation and replication, induce multi-drug resistance, and have a variety of cellular effects, apparently unrelated to their action on tubulin.

B. Specific Aims

- To characterize the growth of several related human breast cancer cell lines in a natural (protein) basement membrane gel (Matrigel) by 31P MRS spectroscopy, and compare the role of estrogen receptor (ER) status in ER positive and ER negative cell lines.
- To observe the effects of hormones, such as estrogen and tamoxifen, on the growth and metabolism of these cell lines, and to investigate the effects of drugs, notably Taxol, on these cell lines with different ER status and hormone dependence.
- To explore the application of 1H MRS spectroscopy to study cancer cell metabolism with water signal suppression and observation of only intracellular signals, and to investigate the resolved proton signals in cancer cell spectra, and in response to the agents described.

2. BODY OF PROPOSAL

A. Cell Perfusion Methods

The use of agarose threads was introduced by Foxall and Cohen (Foxall and Cohen, 1983; Foxall et al, 1984) and is based on the properties of low-temperature gelling agarose (SeaPlaque), that allows mixing of cells with liquid agarose at 37°C, and solidification of the mixture at a lower temperature (for a complete description see Cohen et al, 1989). 1-1.2 ml of cell pellet (ca 2 x 10^8 cells) are mixed with equal volume of 1.8% liquid agarose in phosphate-buffered saline, and immersed in a bath at 37°C for 5-7 min. The mixture is extruded under low pressure through cooled tubing (0.5 mm id) into a 10 mm MRS tube containing growth medium. Using 0.5 mm threads ensures that there is no metabolic compromise, and the cells are viable and in stable energetic status for more than 24 h, while the threads maintain their mechanical strength. Moreover, it was shown that albumin can readily diffuse into the threads (Kaplan et al, 1990a). The gel threads which fill the tube are concentrated at the bottom of the tube by insertion of a plastic insert with the perfusion fittings. The inflow tube is 0.5 mm id, and is placed near the bottom of the tube. The outflow is directed into openings in the insert, and then into an outflow tube. Perfusion rates (0.3-2 ml/min) are maintained by a peristaltic pump, and since the tubes are permeable to air, it is not required to include a gas exchanger in the perfusion system. The perfusion solution should be the buffered growth medium that is most appropriate for the cells studied.

Cells are routinely perfused for periods from 2 to 12 hours, and sometimes much longer if sterility can be maintained (Cohen et al, 1986, Daly et al, 1988. The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that sometimes occurs after ca. 12-24 hours. An initial high level of Pi is indicative of a bad cell sample. If a significant increase of Pi is seen in the first two hours the experiment is usually abandoned. If the Pi/ATP ratio is low, and remains low for this period, the cell sample is considered acceptable (a great deal of experience shows that this parameter is consistent with, but preferable to the exclusion of trypan blue as a measure of cell viability). No other change has ever been seen to occur as long as the cells are adequately perfused. If the perfusion is stopped a rapid increase in the Pi/ATP ratio occurs (Knop et al, 1984). In order to confirm the adequacy of perfusion in any given case the perfusion rate is routinely adjusted to check for no change in the Pi/ATP ratio (rates vary from 2-0.3 ml/min depending on the circumstances).

One of the disadvantages of perfusion studies with agarose threads is the limited proliferative activity inside the threads. The gel thread technique was therefore improved by the use of a basement membrane matrix, matrigel, in which anchorage-dependent cells can multiply while being perfused (Daly et al, 1988). Cell pellet (0.1 ml) is mixed with 2 ml of liquid basement membrane, and the mixture is extruded, as described above for agarose threads, into petri dishes. Cells are allowed to grow in the incubator until the desired densities are reached, and are then transferred to a 10 mm MRS tube. The modification of the perfusion apparatus from one which is used for the agarose threads procedure, includes the insertion of large capacity filters (20 liter) between the peristaltic pump and the tube, which ensure sterility. Thus, the cells can be perfused with fresh medium for prolonged periods (weeks), at a low perfusion rate of 0.5 ml/min, and MRS spectral changes associated with proliferation can be monitored (Daly et al, 1988).

B. Proton NMR Cell Studies

Phosphorus-31 NMR methods have proven to be very informative in the study of cellular metabolism (Cohen et al, 1995) . However, although proton NMR is intrinsically 14 times more sensitive than 31P NMR, very little research has been done in this area (Cohen et al, 1995). There are three main reasons for this, (a) the overlap of the many signals from hundreds of metabolites, (b) the presence of the huge water signal in biological systems, and (c) the overlap of signals from intracellular and extracellular substances, including buffers, metabolites, etc. A few years ago we solved (b) and (c) by the use of diffusion-weighting, whereby the application of timed pulses allowed for the selection of a window for the observation of only slowly moving molecules (Van Zijl et al, 1991). That eliminated all water and extracellular molecules that are moving rapidly, and allowed us to observe the proton signals only from intracellular metabolites.

However, there is still extensive overlap of metabolite signals at 400 MHz, preventing useful application. As was done in the protein field, the way to overcome this resolution problem is to increase the field strength. Thus, it is proposed to extend our studies of cancer cell metabolism to diffusion-weighted proton NMR studies at 600 MHz. The signals one can expect to obtain information from are lactate and phospholipid precursors, such as observed in brain spectra. However, so little work has been done in this area that the expectation of seeing new and interesting phenomena is high.

Another advantage of this approach is that because of the higher sensitivity many fewer cells will be needed to obtain spectra. Since 31P NMR probes use 10 mm tubes and proton NMR probes use 5 mm tubes the overall saving is approximately a factor of 4 in the number of cells needed to obtain good spectra. Added to which, the intrinsic sensitivity should give rise to at least an order of magnitude in the speed to obtain proton spectra (this is without considering the longer relaxation times for 31P nor the extra times required for the diffusion weighting pulses in proton NMR). Since fewer cells are needed in less time the cost of the experiments will go down and many more experiments can be done with fewer cells in less time.

Since all our previous work on 31P NMR has been done with 10 mm tubes, it will be necessary to develop a 5 mm flow through system for holding cells during perfusion. This and other newer applications of microdetection of pH and oxygen tension should result in novel results for this application of high field proton NMR to cancer cell metabolism.

C. Preliminary Results

(i) Growth curves of cell lines in matrigel: The cell lines to be studied in this work are listed in Table 1. In order to determine the nature of the growth of these cell lines in matrigel and the effects of hormones and tamoxifen, a series of experiments were carried out in which cells were counted. The use of matrigel caused problems with automatic counting due to clumping of the cells, and the lack of complete degradation of matrigel by dispase. Ultimately a commercial mixture of dispase and collagenase (Matrisperse) was used. The cells were counted manually. Growth curves of MCF7, MIII and LCC2 cells were determined (Figs 1 and 2). The MIII cell line was selected for its anticipated responsiveness to estrogen and tamoxifen, while the LCC2 cell line was selected for its responsiveness to estrogen only. Upon treatment with these cell lines with these hormonal effectors the growth curves were not considered to be significantly different (Figs 1 and 2). As a consequence no further hormonal growth studies were carried out with these cell lines.

Table 1: Phenotypes of cells used.

Cell Line	ER	[1]Estrogen Dependence	[2]Estrogen Responsivity	TAM Responsivity	ICI 182,780 Responsivity	Metastases
MCF-7	+ ve	dependent	responsive	sensitive	sensitive	no
MCF7/MIII	+ ve	independent	responsive	sensitive	sensitive	yes
MCF7/LCC2	+ ve	independent	responsive	resistant	sensitive	ND[3]
MCF7/LY2	+ ve	responsive	resistant	resistant	resistant	NT[4]
MDA-MB231	- ve	independent	unresponsive	resistant	resistant	yes
MDA-MB435	- ve	independent	unresponsive	resistant	resistant	yes

1 = requirement for E2 to form tumors in nude mice; 2 = respond to E2 by inducing specific genes/mitogenesis; 3 = no data; 4 = non-tumorigenic.

(ii) Baseline spectra of cells: Previously baseline 31P MR spectra of cells lines were obtained in perfused agarose gel threads (Ruiz-Cabello et al, 1993). Cells were routinely perfused for periods from 2 to 12 hours (Cohen et al, 1986; Berghmans et al, 1992). The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that occurs after ca. 12-24 hours. These experiments were repeated multiple times (3 -5) with consistent results. Analysis of the results associated higher levels of PDE and UDPG and lower PC/GPC and PC/PE ratios with the acquisition of hormone independent status (Ruiz-Cabello et al, 1993).

These studies have now been performed for 5 of the six cell lines listed in Table 1 in matrigel. The sixth cell line (LY2) could not be grown satisfactorily during the period these experiments were underway, and will be sent from Dr. Robert Clark's laboratory in Lombardi Cancer Center, Georgetown University, to the laboratory of Dr. Israel Ringel at Hadassah Medical Center, Hebrew University, for completion of this phase of the work. The 31P spectra of each of these cell lines perfused in matrigel are shown in Figs 3-7. A preliminary comparison of the integrals of the resolved and assigned peaks normalized to the β-ATP peak are given in Fig 8. The most significant difference observed is that due to the diphosphodiester peak that varies widely from essentially unobservable in 435 cells to a high level in LCC2. This is listed as UDPS in Fig 8, since the nature of the sugar component is unknown, although from previous work it is highly likely to be N-acetylglucosamine and some N-acetylgalactosamine. It is noted that the main difference in these cell lines is the presence or absence of ER and their consequent responsivity to estrogen.

(iii) Effects of Taxol: In preliminary experiments MCF7 and MD231 cells were incubated with and without Taxol. In order to obtain comparable results it was decided to utilize synchronized cell growth conditions. The cells were starved for 48 hours, after which time using FACS analysis it was found that 80-85% of the cells were in Go phase (Fig. 9). After 48 hours of incubation the cells were harvested and extracted using perchloric acid following previously described conditions (Kaplan et al, 1990). 31P spectra were then obtained on the extracts (Fig. 10 and 11). Differences were seen in the Pi, in the UDPS and the PCr peaks. Normalization and integration of these spectra are currently underway.

3. SUMMARY AND FUTURE PLANS

(a) We have found little effect of estrogen and tamoxifen on the growth curves of MIII and of estrogen on LCC2 cells grown in matrigel.
(b) We were able to carry out comparisons of baseline 31P NMR spectra of 5 cell lines grown in matrigel.
(c) We have noted a significant difference in the UDPS concentrations in 435 and LCC2 cell lines. We are now carrying out extractions and HPLC analysis to determine which of the sugar diphosphodiesters are present. We will follow this up to determine the relationship if any to the ER status of the particular cell lines.
(d) Differences were seen in preliminary spectra of MD231 cells with and without taxol. Whether or not these differences are significant and reproducible remains to be determined. However, if they are, then further studies will be carried out with these and other cell lines embedded in matrigel threads. The use of synchronized cells is a novel feature of this work which will be expanded upon.
(e) No proton NMR studies have been done up to this point. It is intended to carry out these studes at 600 MHz on the new 600 MHz NMR spectrometer at Bar-Ilan University, Ramat Gan, Israel (10 mins drive from the Sheba campus; see letter from Dr. Eva Meirovich).

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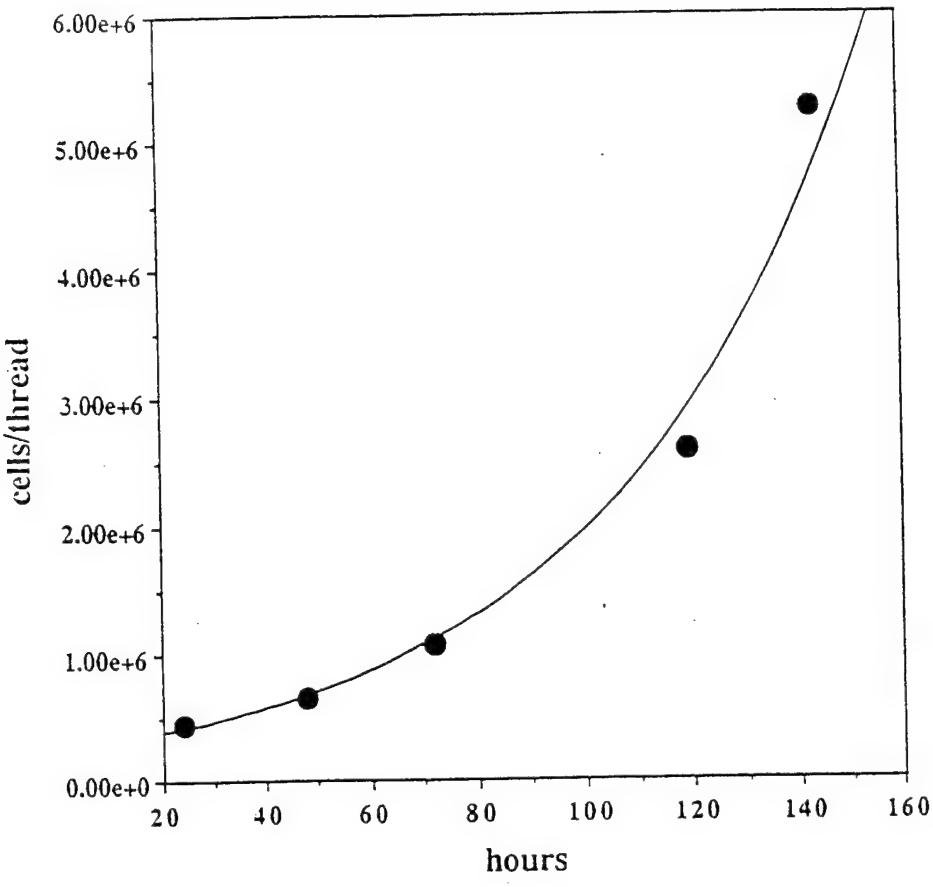


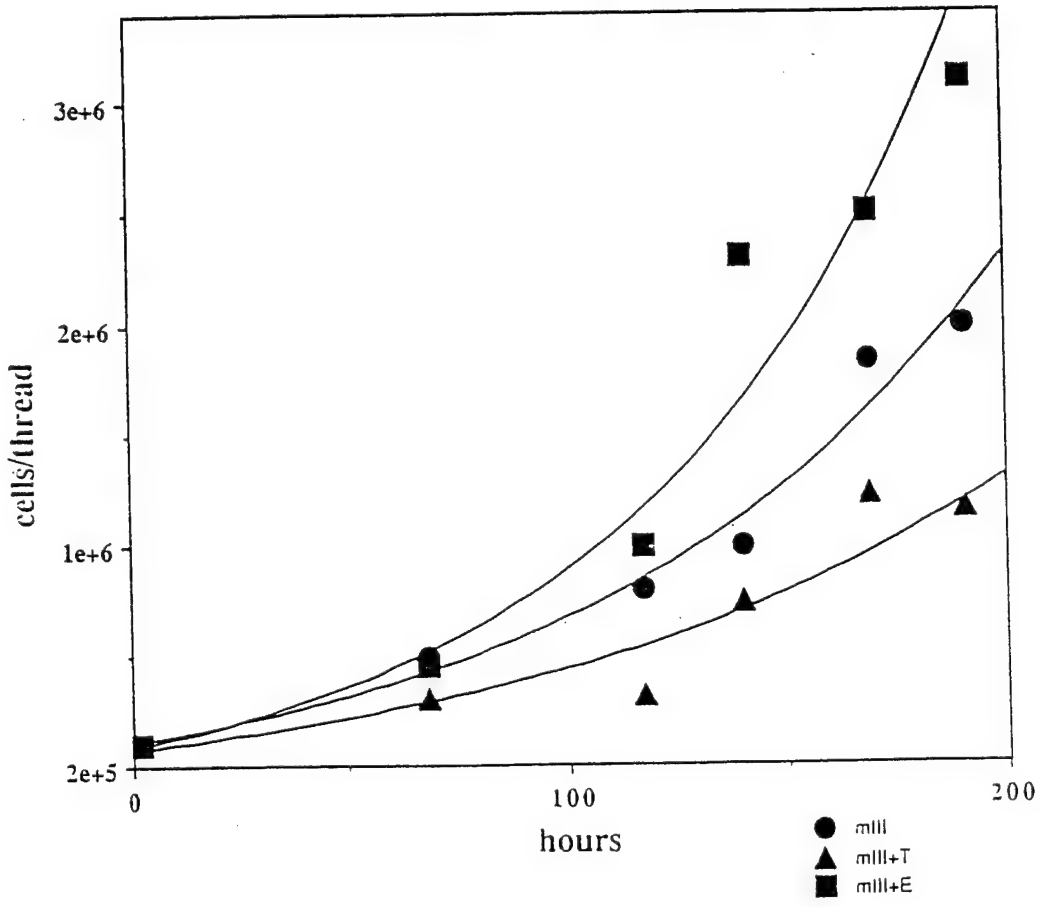


Figure 1. Growth curve for MCF-7 cells growing in Matrigel threads under standard culture conditions (upper) and for MIII cells, also including estradiol (1 nM) and Tamoxifen (0.5 μM). All lines have greater than 0.9 R confidence factor.

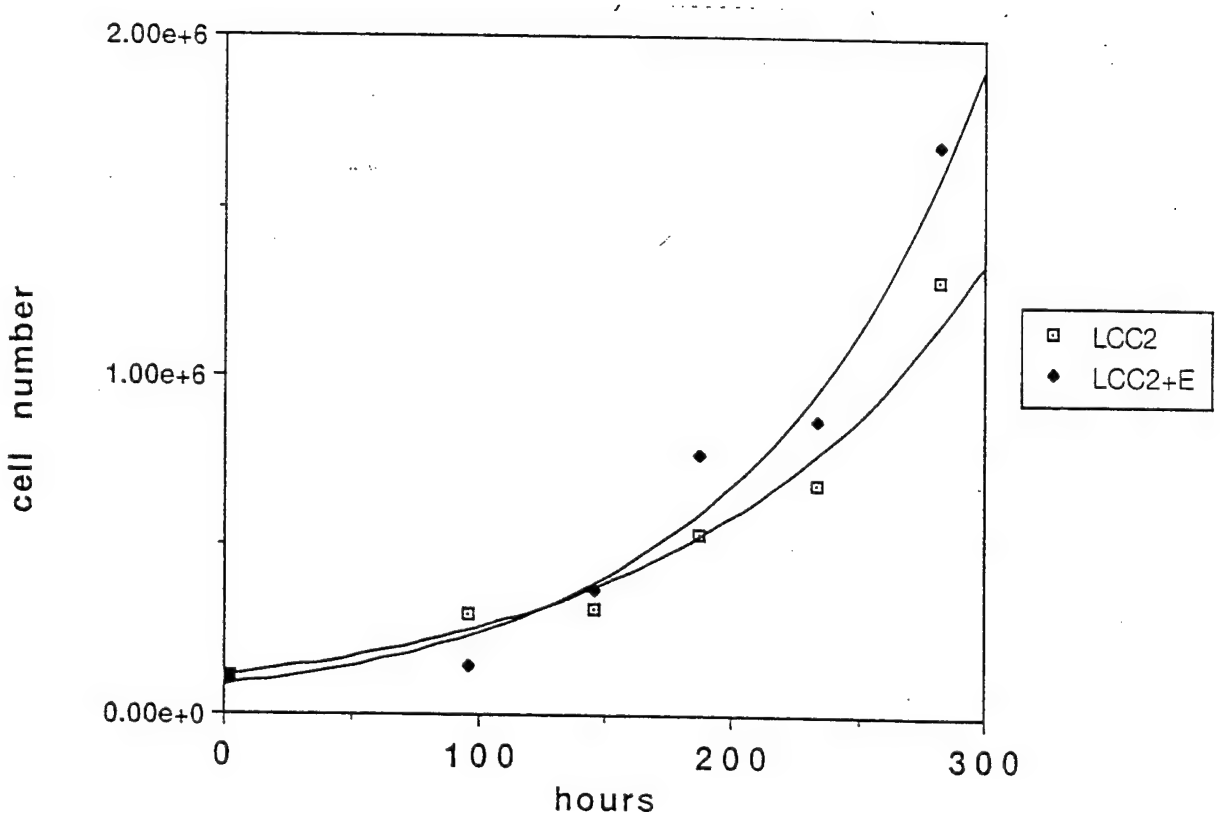


Fig. 2. Growth curves for LCC2 cells with and without estradiol (1 nM). Both lines have greater that 0.9 confidence factor.

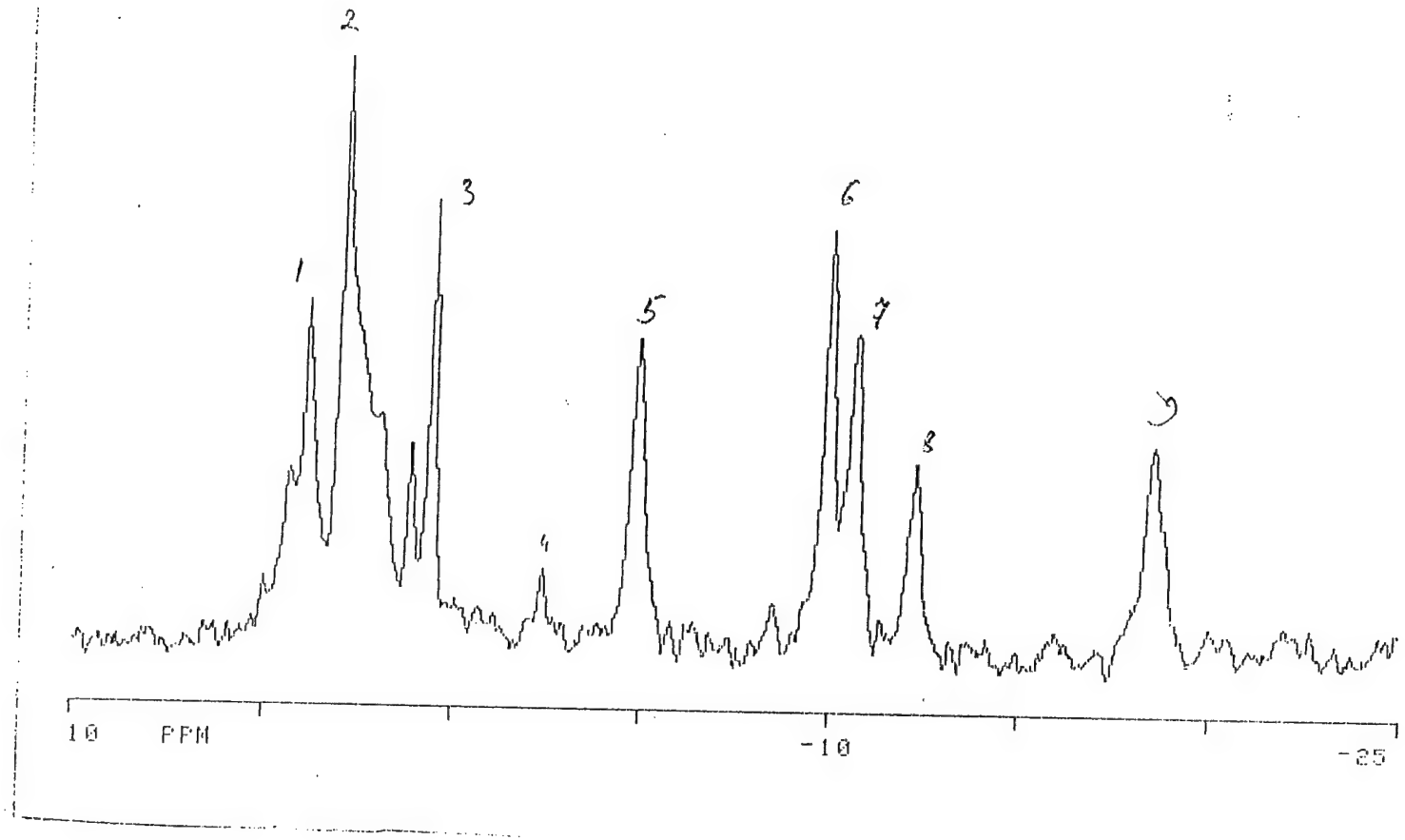


Fig. 3. 31P NMR spectrum of perfused MCF7 cells grown in matrigel threads in the NMR tube.

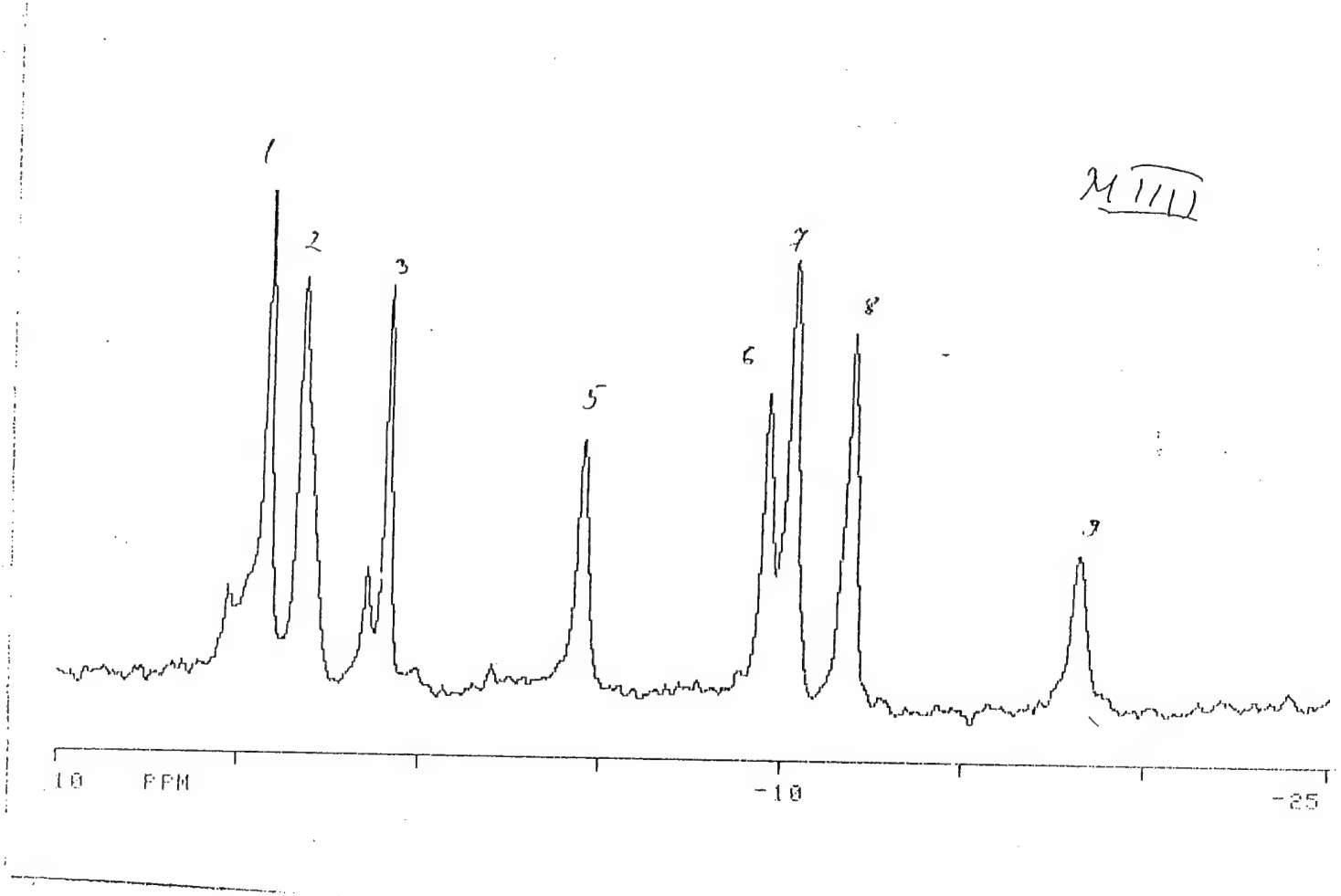


Fig. 4. 31P NMR spectrum of perfused MIII cells grown in matrigel threads in the NMR tube.

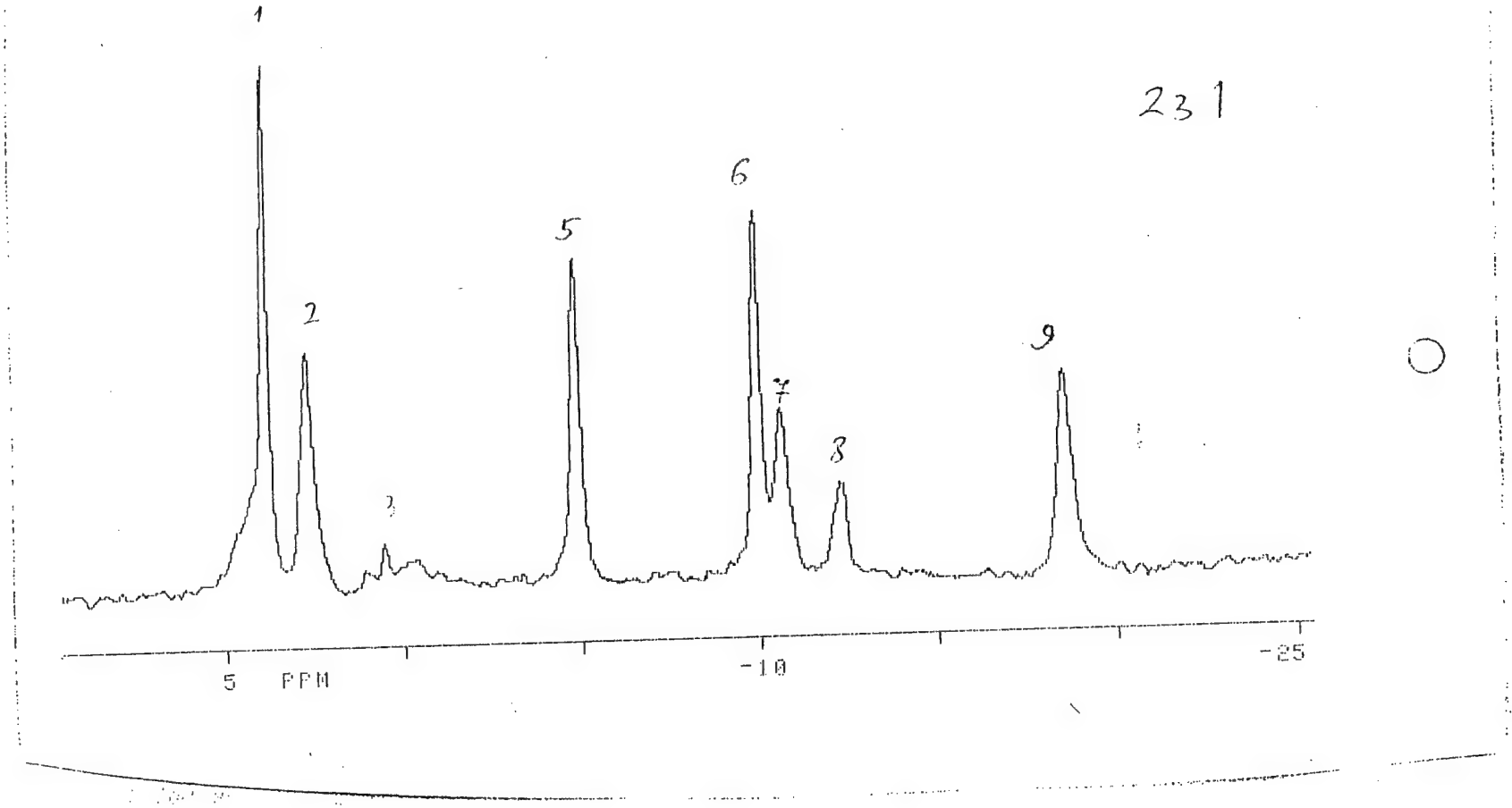


Fig. 5. 31P NMR spectrum of perfused MB231cells grown in matrigel threads in the NMR tube.

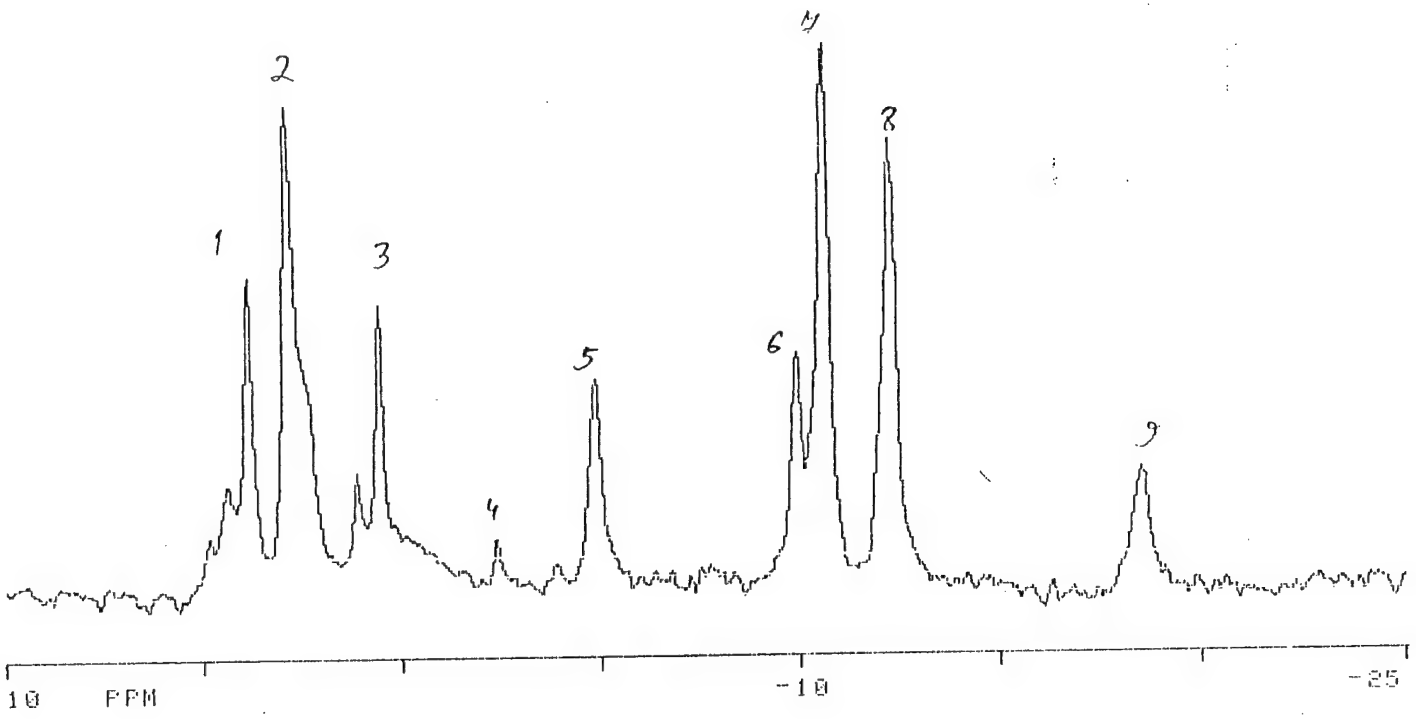


Fig. 6. 31P NMR spectrum of perfused LCC2 cells grown in matrigel threads in the NMR tube.

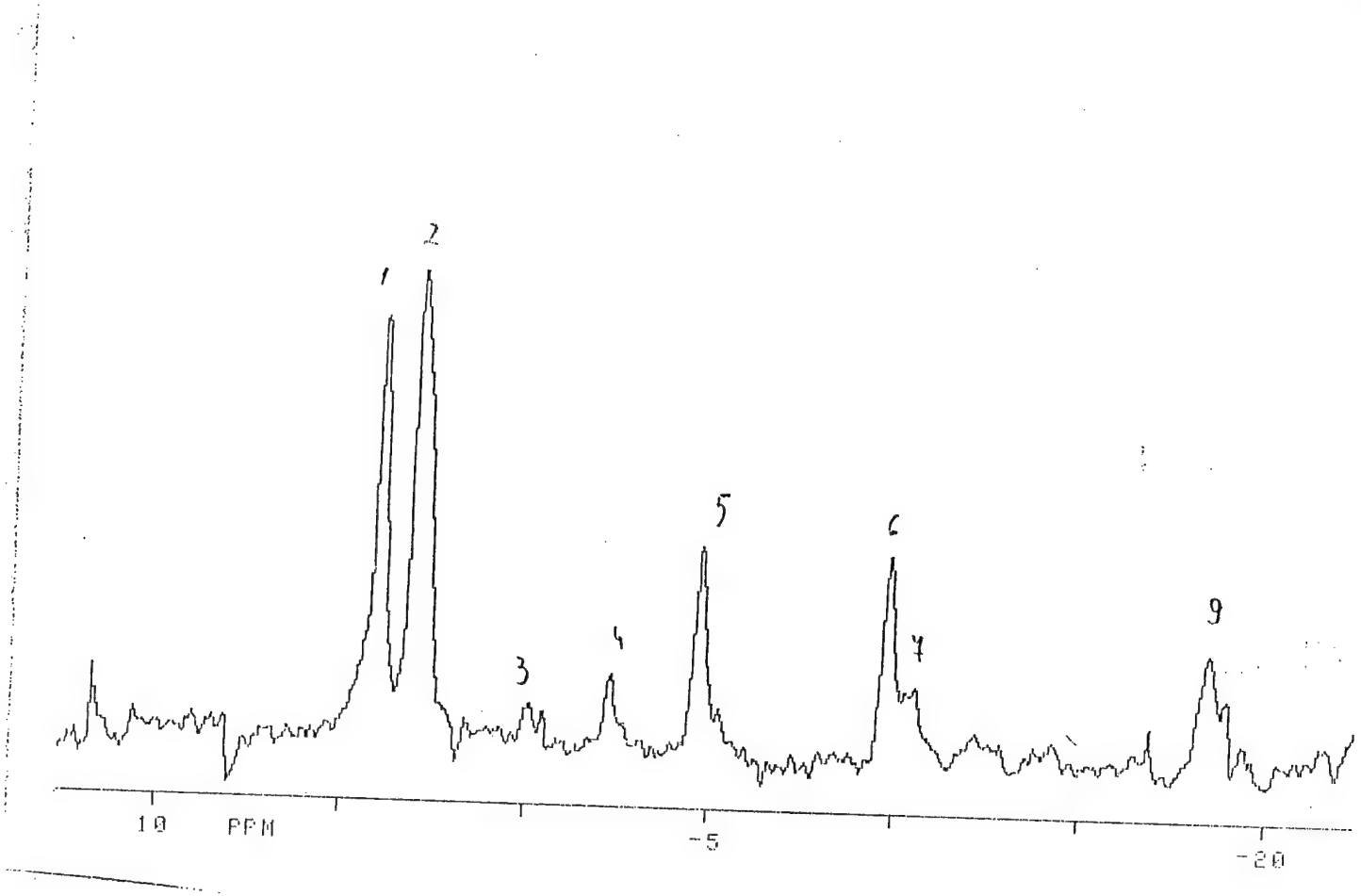


Fig. 7. 31P NMR spectrum of perfused MB435 cells grown in matrigel threads in the NMR tube.

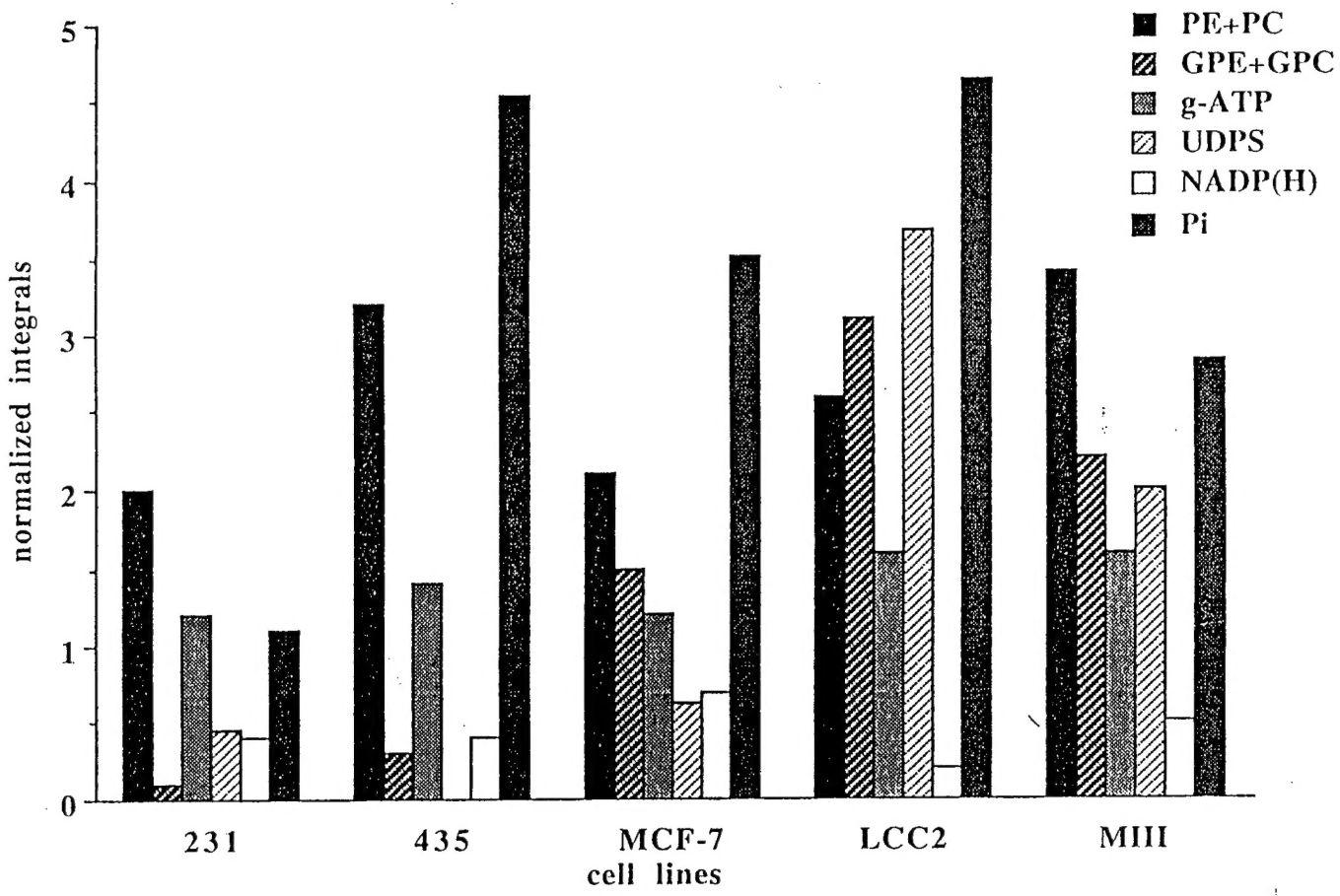
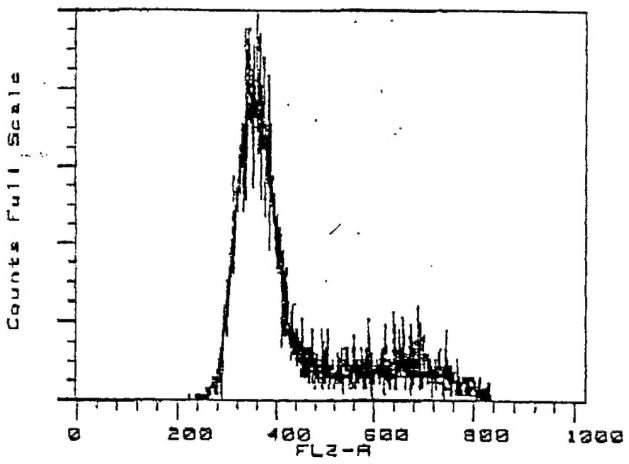


Fig. 8. Integration of 31P NMR signals from cell lines in Figs. 3-7, normalized to the beta-ATP peak.



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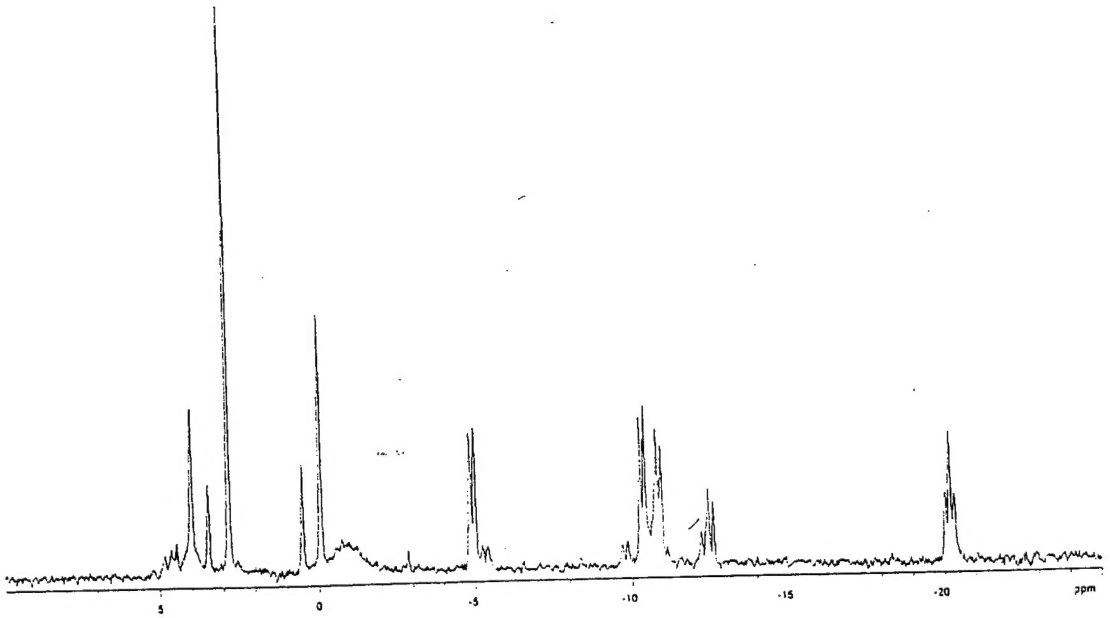
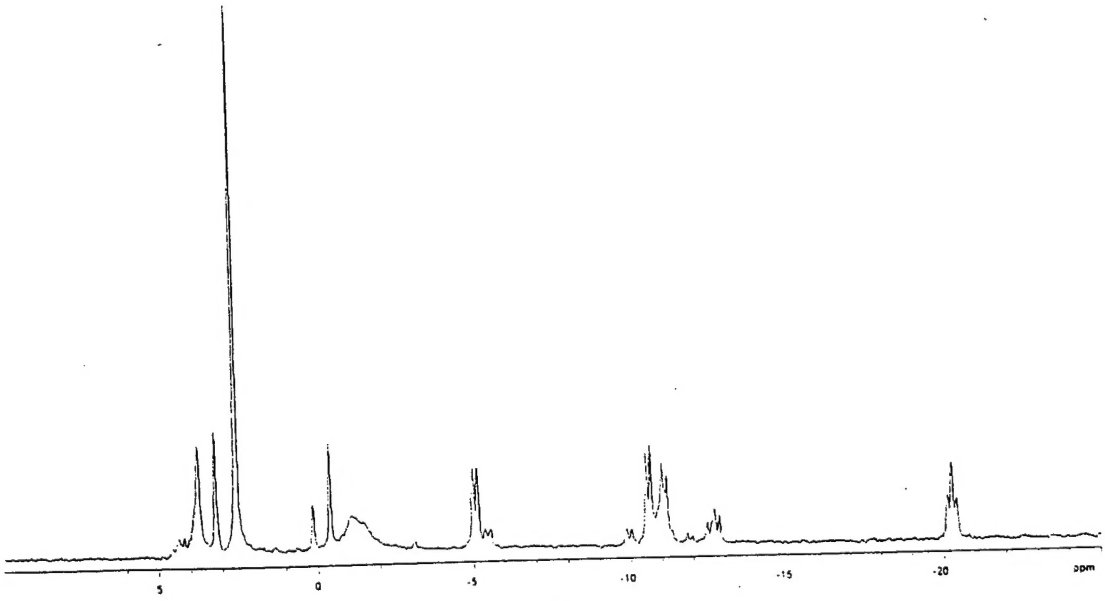
Case Number	:	Gate	: 1
Tissue Type	:	Event Rate	: 138
Preparation	:	Total Events	: 6958
Preparation Date	: 0-NUL- 0	Goodness-of-Fit	: 1.28
Reagents	: FSC-Height FL2-Height FL2-Area	Number S Phase Peaks	: 3
File Name	: #3:061196005	Fit resolution	: 1024
Collection Date	: 6-NOV-96		
Lab	: INTERDEPARTMENTAL UNIT		

Fig. 9. FACS analysis of MCF7 cells starved for 48 hrs, showing ca. 80 in the Go phase; these cells were then used for the experiment in Fig. 10.

MCF-7



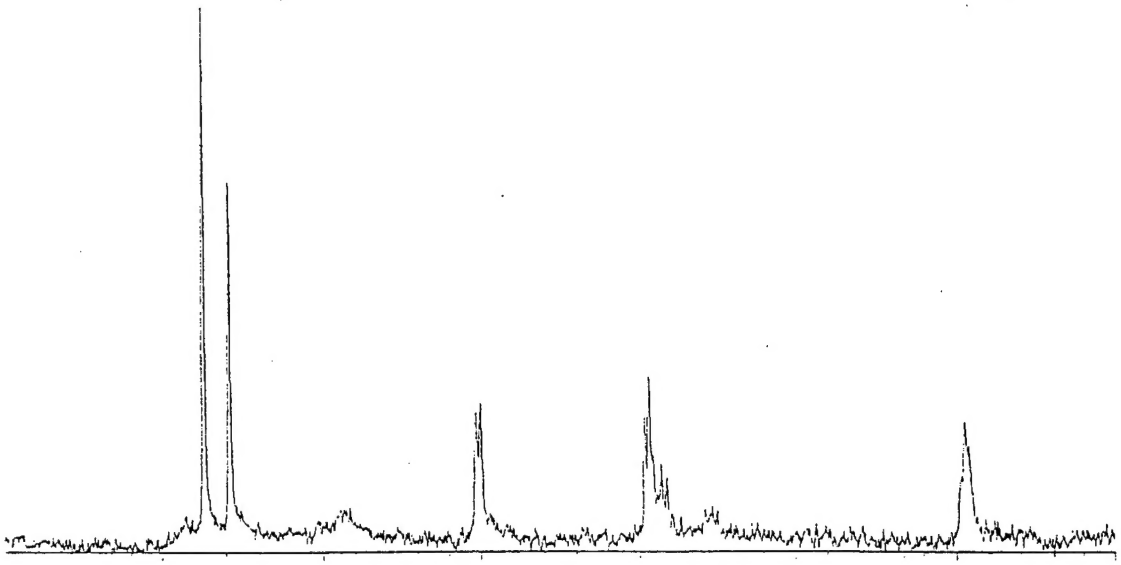
control spectrum



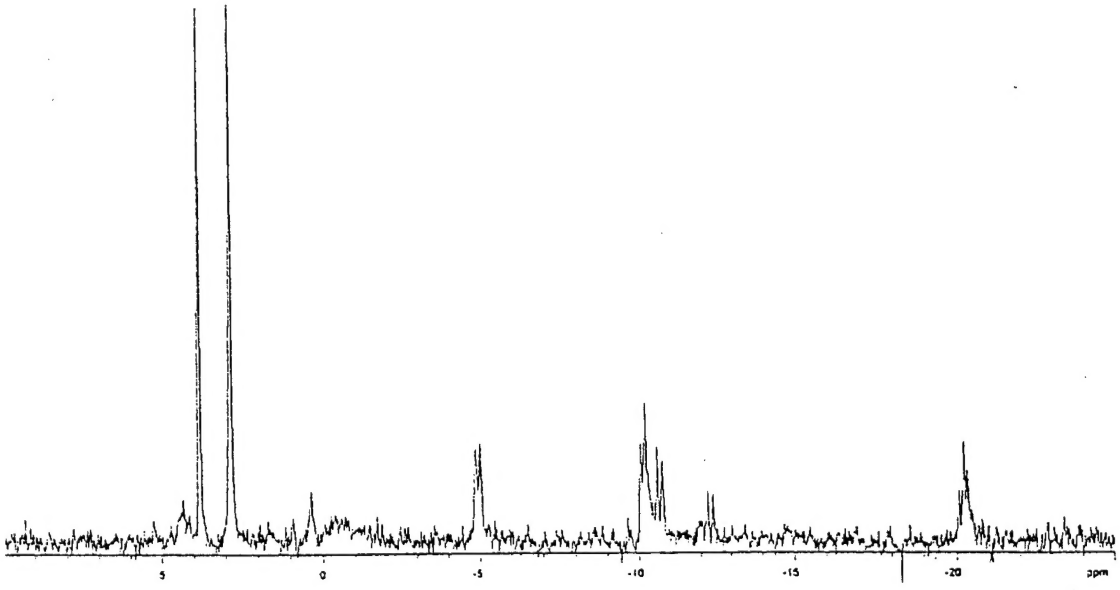
Taxol

Fig. 10. 31P NMR spectrum of extracts of synchronized MCF7 cells incubated alone and with Taxol ($5x10^{-8}$ M) for 48 hours.

MDA-MB-231 cells



control spectrum



Taxol

Fig. 11. 31P NMR spectrum of extracts of synchronized MB231 cells incubated alone and with Taxol (10^{-7} M) for 48 hours.